Parasitism of the Nematode *Criconemella xenoplax* by the Fungus *Hirsutella rhossiliensis*

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ABSTRACT

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Hirsutella rhossiliensis was commonly isolated from dead, surface-disinfested *Criconemella xenoplax* extracted from peach orchard soils. Invasion of living nematodes followed attachment of spores to the nematode's cuticle. After penetration of the cuticle directly beneath the adhering spore, a bulbous infection hypha formed from which secondary hyphae developed. Approximately 25% of inoculated juveniles were penetrated and killed by the fungus under laboratory conditions; adult *C. xenoplax* were penetrated less often. Approximately 50% of stressed adults (120 min incubation at 40 C prior to inoculation) were invaded and killed by the fungus. *H. rhossiliensis* rapidly penetrated and colonized all adults killed by 30 min of incubation at 60 C prior to inoculation.

The plant parasitic nematode *Criconemella xenoplax* (Raski) Luc and Raski (= *Macroposthonia xenoplax* [Raski] DeGrisse and Loof) is one of several factors that contribute to peach tree short life, a serious and complex disease of peaches in the southeastern United States. Presently, there are few methods for effective control of this nematode. In several orchards, populations of *C. xenoplax* have declined unexpectedly even when customary weather patterns and farm practices prevailed (E. I. Zehr, *observation*). Patterns of population decline suggested that reduced numbers may have been caused by parasites or predators. Few parasites or predators of the Criconematidae have been described.

This paper reports the association of a fungus *Hirsutella rhossiliensis* Minter and Brady with *C. xenoplax* in the field and parasitism of the nematode by the fungus in the laboratory.

MATERIALS AND METHODS

Isolation of *H. rhossiliensis* from *C. xenoplax*. In the spring and fall of 1981, soils were sampled in 22 South Carolina peach orchards and one Georgia peach orchard. Subsamples were collected from the canopy edge of 3–10 trees per orchard and combined to form a 1-L sample per orchard. Nematodes were extracted from 500 cm^3 of soil by centrifugal flotation and were examined with a dissecting microscope. Juvenile and adult *C. xenoplax* that appeared to be diseased were mounted in water on a glass slide and observed at ×430 and 1,000 for evidence of parasitism. Specimens were recovered from the temporary mount, surface disinfested 15 sec in a drop of 0.5% NaOCl, and rinsed in a drop of sterile distilled water. A nematode pick consisting of a 1-cm segment of molybdenum wire (90 μm diameter, obtained from Sylvania incandescent light bulbs) heat-fused to the tip of a disposable pasteur pipette was used for aseptic transfer of nematodes. The wire heated and cooled rapidly. After surface disinfestation, nematodes were placed on water agar or cornmeal agar and incubated at 22 ± 2 C. Pure cultures of the fungi growing from nematodes were obtained by transferring hyphal tips from the edge of advancing colonies to water agar, corn meal agar, or potato-dextrose agar. *H. rhossiliensis* was readily isolated by using this procedure. This hyphomycete produces adhesive spores on the tip of flask-shaped phialides (Fig. 1). One isolate of *H. rhossiliensis* (ATCC 46487, IMI 265748) from a South Carolina orchard was used throughout this study.

Parasitism of *C. xenoplax* by *H. rhossiliensis*. *C. xenoplax* was extracted by centrifugal flotation from greenhouse pot cultures that had been started with hand-picked *C. xenoplax* and maintained on peach in autoclaved soil. Gravid females were placed in a 6-cm-diameter petri dish containing 6 ml of sterile distilled water and incubated at 22 ± 2 C. Each nematode subsequently deposited 5–10 eggs over a 2-day period. In 14 days, second-stage juveniles hatched. Using the nematode pick previously described, juveniles were inoculated by touching each nematode to the adhesive spores (~10 spores per juvenile) borne on phialides of *H. rhossiliensis*, which had grown on cornmeal agar at 25 C for 7–14 days. The juveniles were then placed in a 6-cm-

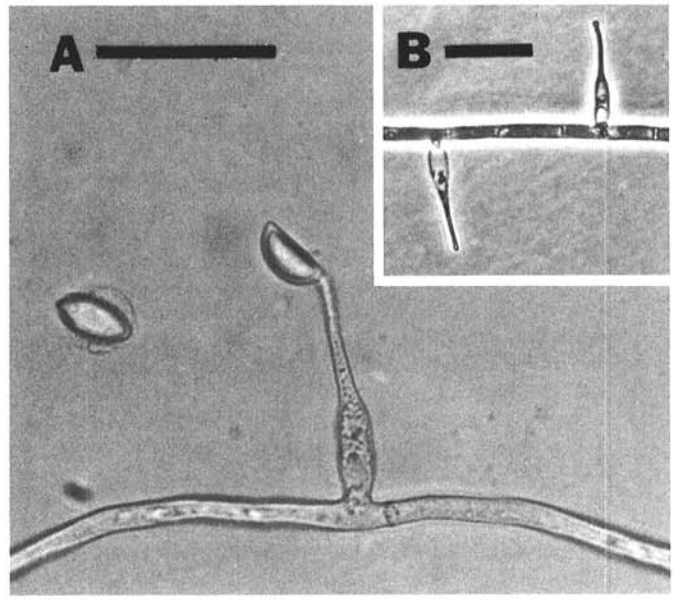

Fig. 1. Phialides and spores of *Hirsutella rhossiliensis*. **A,** with, and **B,** without spores. Bars represent 20 μm.

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diameter petri dish containing 6 ml of sterile distilled water and incubated at 22 ± 2 C. A similar procedure was followed with adult *C. xenoplax* except that the adults were inoculated with ~20 spores per adult. All adults were females; males were not observed or used in this study. Uninoculated juveniles and adults served as controls. Periodically, three or more living nematodes (nematodes were considered alive if they were moving or if they moved after being gently touched with a nematode pick [3]) from each treatment were mounted in water on a glass slide and were observed at ×430 and 1,000 with bright-field and phase-contrast microscopy. The preparations were allowed to dry slightly to facilitate observation of fungal hyphae within the nematodes.

To quantify the number of individuals parasitized, 20–30 juveniles and 20–30 adults were inoculated with spores of *H. rhossiliensis* as previously described and incubated in sterile distilled water at 22 ± 2 C. Uninoculated juveniles and adults served as controls. At 5 and 10 days after inoculation, 10–15 nematodes per treatment were examined at ×430 and 1,000 and the percent parasitized and dead nematodes determined. This experiment was performed four times. Data were subjected to analysis of variance with single degree-of-freedom comparisons.

Effects of heat treatment. Adult *C. xenoplax* were killed by heating at 60 C for 30 min and then inoculated with either one or 20 spores of *H. rhossiliensis* per nematode as previously described. Inoculated and uninoculated (control) specimens were incubated in sterile distilled water at 22 ± 2 C. The percent colonized nematodes per 10–15 specimens was determined 5 and 10 days after inoculation. The experiment was performed four times.

In another experiment, adult *C. xenoplax* were incubated for 0, 60 and 120 min at 40 ± 1 C in 1 ml sterile distilled water, stored overnight (15 hr) at 22 ± 2 C, and then half were inoculated with *H. rhossiliensis* (~20 spores/nematode) as previously described. The percent of dead and fungal-penetrated nematodes per 20–25 specimens was determined 5 days after inoculation. The experiment was performed five times. Data were subjected to analysis of variance with single degree-of-freedom comparisons.

Parasitism of *C. xenoplax* eggs by *H. rhossiliensis*. A small block of cornmeal agar containing *H. rhossiliensis* was transferred to the center of a water agar plate and incubated for 5 days at 25 C. Five gravid females, previously rinsed in sterile distilled water, were transferred to the border of the colony and the plate was then incubated at 22 ± 2 C. Females deposited 5–10 eggs per female in and on the agar in the next 2 days. Eggs were observed daily for 2 wk. Eggs deposited in sterile water agar were controls. The experiment was performed twice.

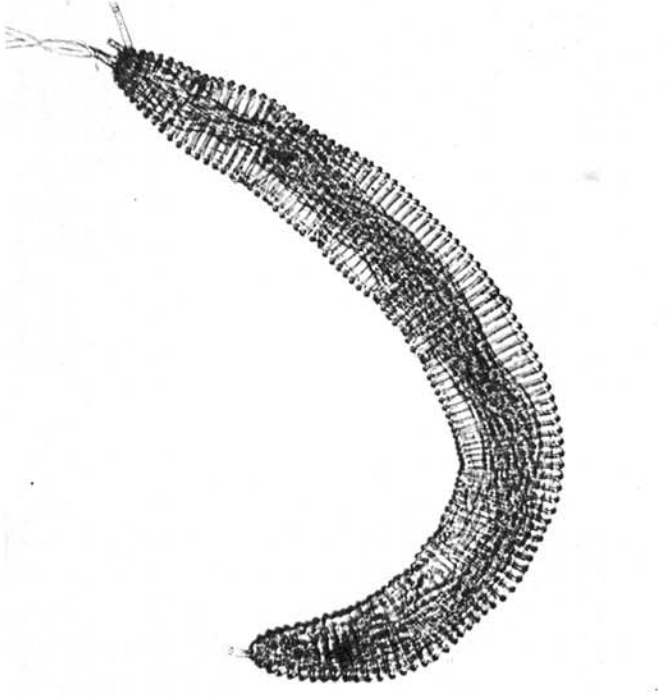

Fig. 2. Typical *Criconemella xenoplax* from which *Hirsutella rhossiliensis* was isolated (×160).

RESULTS

Isolation of *H. rhossiliensis* from *C. xenoplax*. Ten of the 23 orchard soils surveyed contained dead juvenile and adult *C. xenoplax* with brown heads and distorted, hyphae-filled bodies (Fig. 2). Hyphae often extended from the head and the tail and occasionally bore flask-shaped phialides without spores. As many as 40, but usually 1–10, spores were observed adhering to the nematode's cuticle, usually near the head (Fig. 3A). In one sample containing 1,000 *C. xenoplax* per 100 cm^3 of soil, 900 had brown heads and hyphae-filled bodies as described. Living specimens with spores attached were also observed.

A fungus with septate hyphae and flask-shaped phialides (length = 25–35 μm) bearing oval to ellipsoid spores (one spore per phialide) was readily isolated from these specimens (Fig. 1A and B). Spores (~8 × 4 μm) often resembled orange segments in shape and were usually covered by a nonpigmented, adhesive material. The fungus grew slowly on water, cornmeal, potato-dextrose, carrot juice, V-8 juice, and other agar media. Phialides, borne singly and perpendicular to the hypha, were always aerial; ie, they never developed in water or agar. The spores readily detached and adhered to the nematode's cuticle when *C. xenoplax* juveniles or adults touched them (Fig. 3B). A. Y. Rossman and D. W. Minter identified this fungus as *H. rhossiliensis* Minter and Brady.

Parasitism of *C. xenoplax*. Three to 4 days after inoculation, penetration through the cuticle and directly beneath the adhering spore was observed in six of 12 living juveniles that had been inoculated. Penetrations were observed in anterior, posterior, and middle regions of the nematode. After the cuticle was penetrated, a

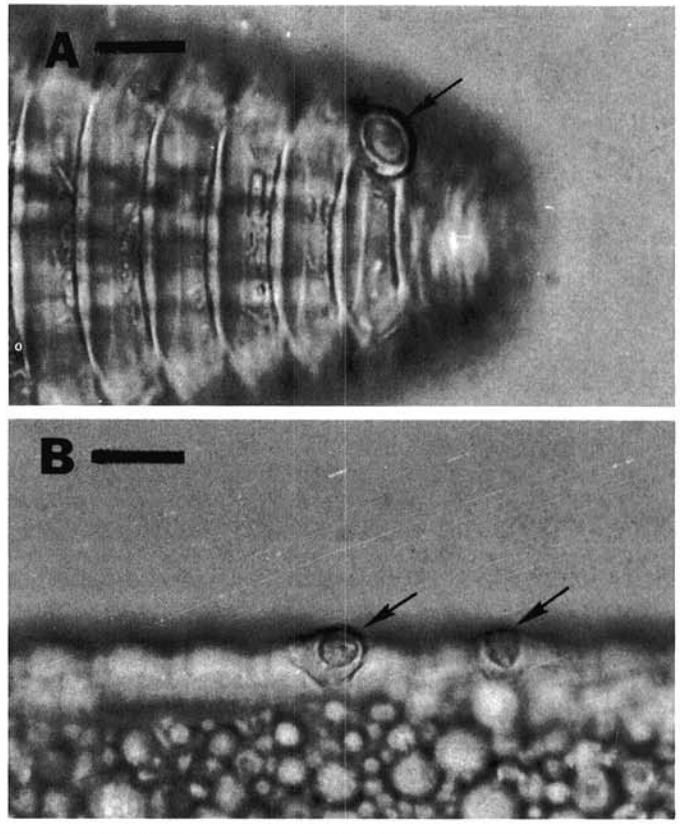

Fig. 3. *Hirsutella rhossiliensis* spores (arrows) adhering to cuticle of *Criconemella xenoplax*. **A,** Nematode recovered from soil with spore attached. **B,** Nematode artificially inoculated. Bars represent 10 μm.

bulbous infection hypha (4–7 μm in diameter) formed (Fig. 4A and B). One or two secondary hyphae (2–4 μm in diameter) developed from the infection hypha. Tissue degeneration and nematode death were associated with extension of the secondary hyphae (Fig. 4C and D). After colonization, hyphae emerged from the dead nematode, usually from the head and tail. If infected nematodes were placed on a moist surface (agar or filter paper), the hyphae produced phialides and spores almost immediately upon emergence from the nematode. Infection of living adults occurred, but that was more difficult to observe due to their greater diameter. Colonized nematodes often adhered to the bottom of the petri dish or floated to the surface of the water.

TABLE 1. Infection and mortality of *Criconemella xenoplax* inoculated with *Hirsutella rhossiliensis*[a]

Nematode stage	Inoculated	5 Days		10 Days	
		Penetrated[b]	Dead[c]	Penetrated[b]	Dead[c]
Juvenile	Yes	21	17*	26	32*
Juvenile	No	0	5	0	7
Adult	Yes	7	7	6	6
Adult	No	0	3	0	3
Adult[d]	Yes	100	100	100	100
Adult[d]	No	0	100	0	100

[a] Nematodes were touched to *H. rhossiliensis* spores (10 spores per juvenile, 20 spores per adult) and incubated in sterile distilled water for 5 or 10 days. Values at 10 days are independent of values at 5 days. Each value is the mean of four replications (10–15 nematodes per replication).
[b] Percent nematodes containing *H. rhossiliensis* hyphae.
[c] Percent dead nematodes. Means followed by asterisk are significantly greater than those of uninoculated control ($P = 0.05$) as determined by analysis of variance with single degree of freedom comparisons.
[d] Killed by heating at 60 C for 30 min in 1 ml of water.

Significantly ($P = 0.05$) more juveniles died in the inoculated than uninoculated treatment within 5 or 10 days after inoculation, but the number of dead adults was not significantly affected by inoculation (Table 1). The mean number of spores per inoculated juvenile and adult was 10 and 20, respectively. Uninoculated specimens had no spores adhering to the cuticle, were not penetrated, and showed no other signs of fungal activity.

Effects of heat treatment. All heat-killed adult *C. xenoplax* inoculated with 20 spores per nematode were invaded by the fungus 5 days after inoculation (Table 1). Forty percent of the heat-killed *C. xenoplax* contacted by only one spore were invaded 10 days after inoculation. Uninoculated, heat-killed *C. xenoplax* were not invaded by fungi.

In three of five experiments involving sublethal heat treatment, nematodes exposed to 40 C for 60 or 120 min were immobilized for 2–3 days. Within 5 days after inoculation, most nematodes had recovered. However, 53% of those heated to 40 C for 120 min prior to inoculation were invaded and killed by the fungus (Fig. 5). In a fourth experiment, exposure to 40 C for 120 min killed all of the nematodes and a 60-min exposure to 40 C resulted in high mortality (64%) of inoculated nematodes. Less than 10% mortality occurred among nonheated nematodes and nematodes heated for 60 min but not inoculated. In a fifth experiment, fungal invasion and

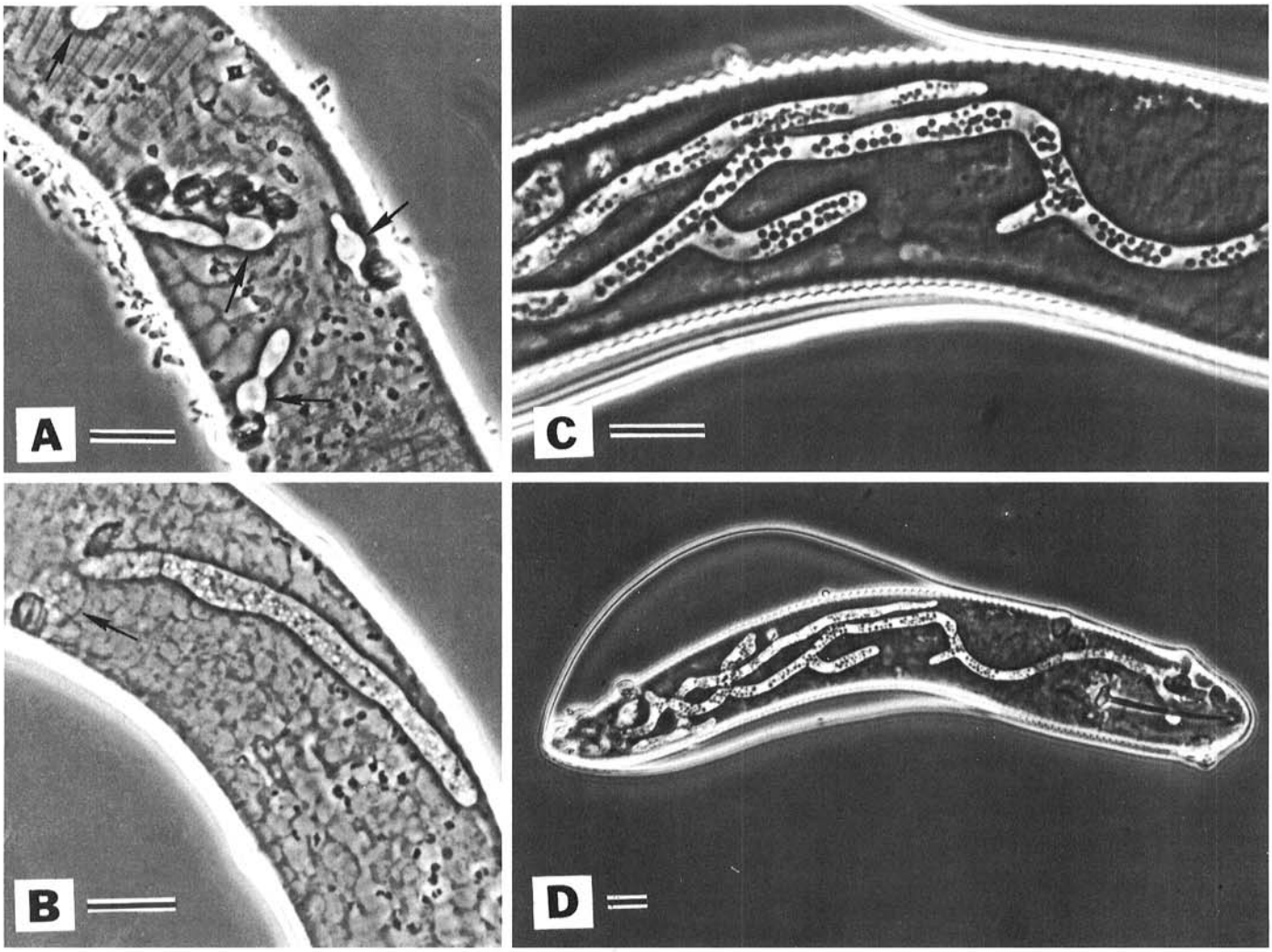

Fig. 4. Infection of juvenile *Criconemella xenoplax* by *H. rhossiliensis*. **A** and **B,** Living juveniles containing bulbous infection hyphae (arrows) from which secondary hyphae are developing. **C** and **D,** Dead juvenile containing secondary hyphae. Bars represent 10 μm.

subsequent death was not evident until 10 days after inoculation when 29% of nematodes exposed to 40 C for 120 min were killed. Mortality after shorter time exposures at 40 C and in uninoculated controls was less than 9%.

Parasitization of *C. xenoplax* eggs. Although most eggs were near hyphae of *H. rhossiliensis*, no parasitism was observed. Eggs developed normally and hatched in approximately 2 wk whether *H. rhossiliensis* was present or absent.

DISCUSSION

In 1980, Minter and Brady (2) described *H. rhossiliensis* isolated from soil in Wales. Several months later Sturhan and Schneider (4) described *H. heteroderae* isolated from soilborne nematodes in Germany. The descriptions, illustrations, and micrographs of *H. rhossiliensis* and *H. heteroderae* suggest that these species are synonymous. Until this question is addressed in the mycological literature, we are using the name *H. rhossiliensis* because the description by Minter and Brady preceded that of Sturhan and Schneider.

Sturhan and Schneider (4) isolated *H. heteroderae* from *Heterodera humuli* juveniles and reported the fungus to be pathogenic to certain species of *Heterodera, Globodera, Pratylenchus, Meloidogyne, Ditylenchus,* and *Aphelenchus*. No Criconematidae were tested. Our finding is the first report of *H. rhossiliensis* in the United States and the first report of an association of *C. xenoplax* and *H. rhossiliensis*. Another species of *Hirsutella* (*H. thompsonii*) that parasitizes mites is currently being investigated as a biological control agent (1).

Our survey of 23 orchards indicates that *H. rhossiliensis* is often associated with *C. xenoplax* in South Carolina peach orchards. In some soils the population density of *H. rhossiliensis* is quite high; over 500 *H. rhossiliensis*-colonized *C. xenoplax* per 100 cm^3 soil have been recovered from two orchards (*unpublished*). These counts are probably underestimations. Infected nematodes that adhere to the soil or float on water are likely to be lost in the extraction process. The number of *H. rhossiliensis*-invaded nematodes in the soil can be determined only by placing nematodes on a suitable medium and allowing the fungus to grow out. The symptoms (brown head and distorted body) of nematodes containing this fungus are not diagnostic because a *Verticillium* sp. sometimes is recovered from such nematodes. The presence of emergent hyphae bearing *H. rhossiliensis*-type phialides can be considered diagnostic, because nematodes with these signs have consistently yielded *H. rhossiliensis* when placed on agar media. Unfortunately, the emergent hyphae are often broken off or damaged when nematodes are extracted from the soil.

Although the impact of *H. rhossiliensis* on the population density of *C. xenoplax* is not yet known, the fungus clearly is capable of parasitizing this nematode; penetrated and infected living juveniles and adults were observed. Although juveniles were invaded more often than adults in laboratory tests, field samples always contained more colonized adults than colonized juveniles. This might reflect either a greater extraction efficiency for colonized adults or different environmental conditions in the field than in the laboratory. The increased invasion of adults exposed to sublethal heat suggests that stress may influence the degree of parasitism occurring in the field. The effect of sublethal nematicide concentrations, low soil water potential, starvation, or other stresses on parasitism of *C. xenoplax* by *H. rhossiliensis* should be investigated.

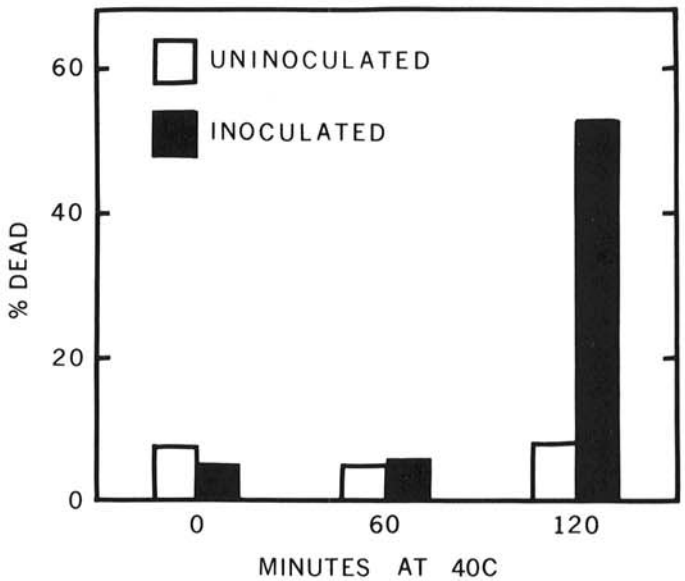

Fig. 5. Mortality of *Criconemella xenoplax* exposed to sublethal heat treatment prior to inoculation with *Hirsutella rhossiliensis*. Adult nematodes were incubated in sterile distilled water at 40 C for 0, 60, or 120 min; inoculated with 20 spores of *H. rhossiliensis* per nematode; and incubated in sterile distilled water for 5 days at 22 ± 2 C. Uninoculated nematodes were controls. Values are means of three replications (20–25 nematodes per replication). The mortality of inoculated nematodes exposed to 40 C for 120 min was significantly greater ($P = 0.05$) than in other treatments. Death of inoculated nematodes was always associated with fungal invasion. Death of uninoculated nematodes was not associated with fungal invasion.

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